IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of

Maria Elena FERRERO — Conf. 3886

Application No. 10/589,621 — Group 1623

Filed October 6, 2006 — Examiner L. Crane

THE USE OF O-ATP FOR THE TREATMENT OF DISEASES INVOLVING ANGIOGENESIS

DECLARATION UNDER RULE 132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Maria Elena Ferrero, hereby declare as follows:

My relevant background and experience are set forth on the attached c.v. I make this declaration in support of the present application, and to provide evidence in rebuttal of several contentions set forth in the outstanding Official Action.

In support of this position, we present experimental data showing the effectiveness of o-ATP as antiangiogenic and antiapoptotic agent *in vitro* and *in vivo* models.

The antiangiogenic effects of oATP were demonstrated in experiments where either non-stimulated or TNFα-stimulated PBMC transendothelial migration was inhibited by the addition of oATP. In a second set of experiments, oATP was shown to inhibit cell-proliferation by inducing apoptosis in RMA (lymphoma) cells in

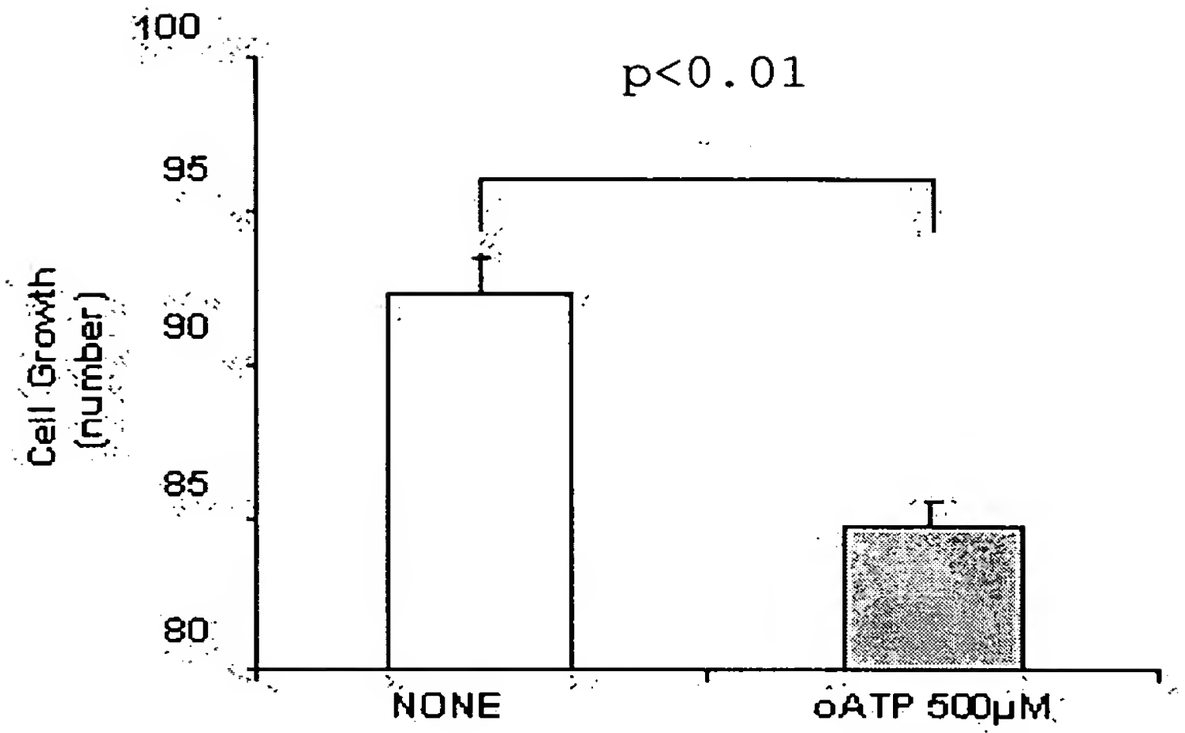
culture. Similar results were obtained *in vivo*, where oATP proved able to reduce tumor growth in mice injected with RMA cells.

These results are predictive of an antitumor activity, especially against lymphoma and leukemia, as recited in claim 4.

HUVEC were isolated from human umbilical cord by collagenase treatment and cultured in 1% gelatine-coated flasks using endotoxin-free Medium 199, containing 20% heat-inactivated fetal bovine serum, 1% bovine retinal-derived growth factor, 90 microg/ml heparin, 100 IU/ml penicillin and 100 microg/ml streptomycin. All experiments were carried out with HUVEC at the passage 1-4.

We used oATP at the concentration of 500 microM. HUVEC were treated with oATP over night, washed and fixed with glutaraldeide 2% in PBS. The cells were coloured with crystal violet 0.1%, washed and dried. The dye solubilisation was performed with acetic acid 10% and the absorbance was measured spectrophotometrically at 595 nm, using a microplate reader. The optical density was proportional to the number of cells. As reported in the Figure 1, cell growing number is significantly reduced by 500 microM oATP treatment (mean±SEM of 7 experiments).

Figure 1



VEGF is a prototypic angiogenetic factor which induces endothelial cell proliferation, angiogenesis and capillary permeability. The latter is evidenced by the assay of transendothelial migration. It is known that VEGF increases transendothelial migration. Migration assay was performed using a Transwell double chamber system (5 micrometer polycarbonate membrane). HUVEC (5 x 10^4 cells/well) were seeded on the filter, in the presence or absence of TNF□ (200 U/ml) and oATP 500 microM. Freshly obtained PMBC (peripheral blood mononuclear cells) by means of fycoll from buffy-coat were added in the upper compartment and allowed to migrate over night to the lower chamber which contained RPMI and 10% fetal calf serum. At the end of the culture, migrated cells were recovered from the lower chamber and counted Figure 2 shows the transendothelial migration

assay. The measure of migrated PBMC through a HUVEC monolayer indicates that the addition of TNFα (200 U/ml) significantly increases the number of migrated PBMC, expressed as percentage over basal, whereas the addition of 500 microM oATP significantly reduces the TNFα-induced transendothelial migration. Data represent mean±SEM of 7 experiments.

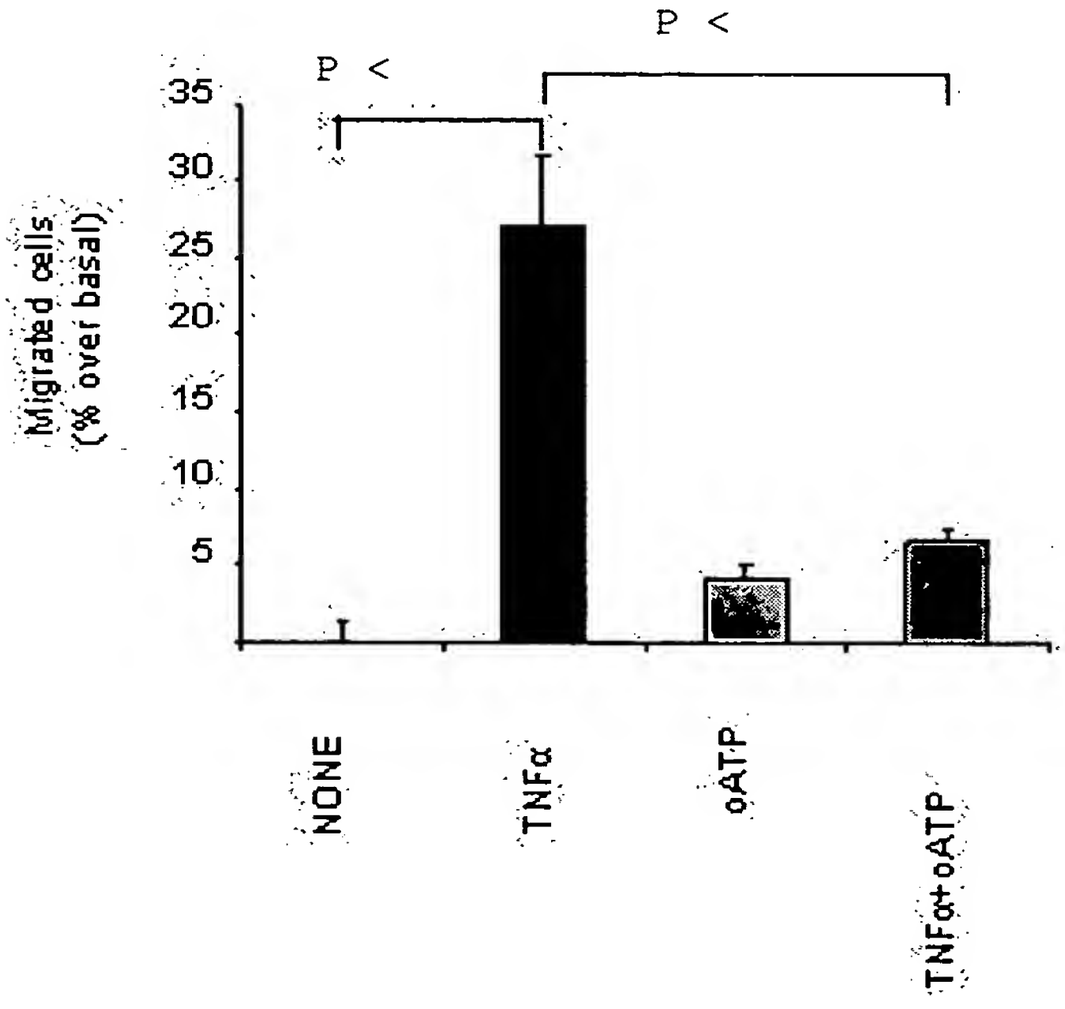


Figure 2

In another series of experiments, we tested the possible direct effect of oATP on tumor cell growth. We used, both in vitro and in vivo, the RMA cells.

In vitro experiments

RMA cells were derived from the Rausher leukemia virus-induced mouse T-cell lymphoma RBL-5 of B6 origin.

Cell isolation and cultures

RMA wild type (wt) murine lymphoma T cell line (ATCC) was grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 microg/ml streptomycin.

Lymphocytes were obtained from a buffy-coat by a fycoll gradient and depleted from monocytes by adherence.

Lymphocytes were activated with Phytohaemoagglutinin A mitogen (PHA).

Cell apoptosis assay

Apoptosis was evaluated by FACS analysis, after staining with annexin-V FITC-conjugate to show the exposure of phosphatidyl serine on the external side of plasma membranes, and with propidium iodide (PI).

Two thousand RMA wt cells and lymphocytes were treated with or without different concentrations of oATP for 24 and 48 hours. The cells were then washed with PBS with Ca++ and Mg++ and stained.

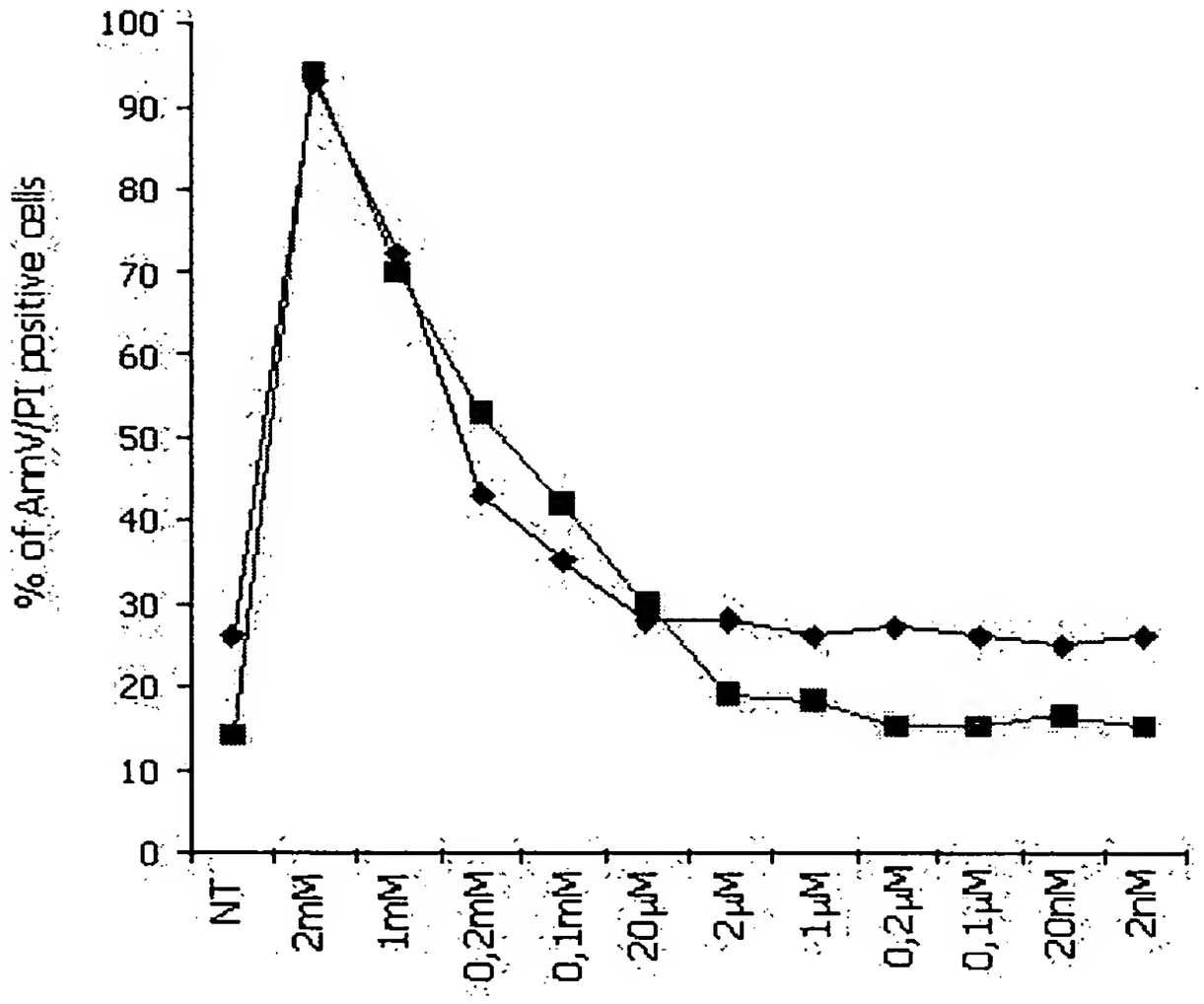
Ten thousand cell/sample were analysed and the percentage of annexin V/PI positive cells was calculated with FCS-express software (DE NOVO Software).

Cell cycle analysis

RMA wt cells were treated with different concentrations of oATP for 24 and 48 hours, then fixed for at least four hours in ice-cold 70% ethanol. Cells were stained with a solution containing NP-40, RNase and PI and cell cycle distribution was detected using FACScan (Becton Dickinson, San Jose, CA) and analyzed with FCS-express software.

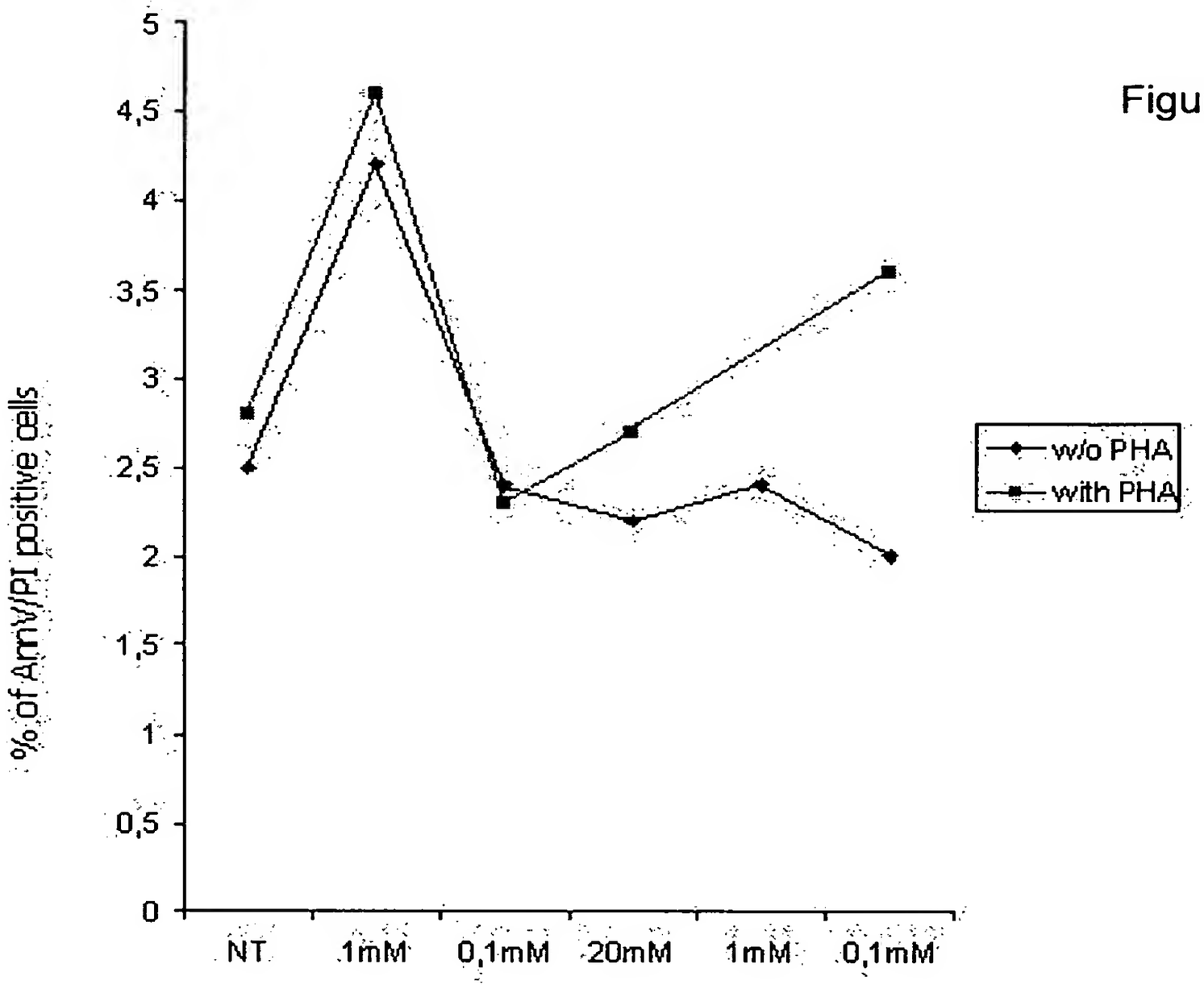
The obtained results indicate that oATP, at high concentrations, is able to induce apoptosis of RMA cells and not of lymphocytes.

Figure 3



Sensitivity of RMA wild type cell line to different concentrations of oATP evaluated as percentage of AnnexinV-PI positive cells at 24 and 48 hours of treatment.

Figure 4



Sensitivity of lymphocytes from buffy-coat (PMBC), activated or not with PHA, at different concentrations of oATP, evaluated as percentage of AnnexinV-PI positive cells at 24 hours of treatment.

The values of the cell cycle progression are reported in Table 1.

Table 1

oATP24h	%subG1	%G0/G1	%S	%G2/M
NT	8	42	32	18
1mM	75	16	6	3
0,1mM	31	44	20	5
1µM	23	38	29	10
0,1µM	9	48	24	19

oATP48h	%subG1	%G0/G1	%S	%G2/M
NT	10	50	24	16
1mM	24	44	25	7
0,1mM	14	33	33	20
20µM	8	50	23	19
1µM	3	52	19	26
0,1µM	4	50	24	22

Cell cycle progression of RMA wt cell line treated with different concentrations of oATP for 24 or 48 hours

Comment

At high doses of oATP, there is a high mortality of RMA wt cells by apoptotic mechanism, as demonstrated by sub G1 peak, in accordance with the percentage of AnnexinV/PI positive cells (Figure 3). Concomitantly, surviving cells loss partially the capacity to entry in S and/or G2/M Phases of the cell cycle, mainly at 24 hours of treatment.

When the concentration of the oATP decreases, cells display a trend similar with the control one.

At high dose of oATP (1mM) and 48 hours of treatment, there is a paradoxical decrease in the percentage of cell death, assessed by sub G1 peak, at variance with the percentage of AnnexinV/PI positive cells which strongly increases (Figure 3). This could depend on the proliferation of cells escaped from the treatment.

To note that, at low doses, there is increase of the percentage of cells in the G2/M phase of the cell cycle.

In vivo experiments

RMA wt cells were maintained in RPMI 1640 medium, supplemented with fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine (complete medium).

C57BL/6 female mice weighing about 18-20 g (8 week old) were used. The cells were washed twice with 0.9% NaCl and subcutaneously injected in each mouse in a volume of 100 microliters containing $7x10^4$ cells. After 10 days from RMA cell injection, we treated the mice with oATP. Five groups of mice were studied (each of 7 mice): 1) controls, untreated; 2) locally (subcutaneously) (sc) daily treated with 0.5 mg of oATP; 3) locally (sc) daily treated with 1 mg oATP; 4) locally (sc) treated with 1 mg oATP for 3 days only. 5) intraperitoneally daily treated with 1 mg oATP.

The mice were observed until the 20th day from the tumor inoculation: they were weighed daily and the size of the tumor mass was daily measured.

The tumor mass did not significantly grow in the groups 2 and 3 for ten days from the beginning of the treatment (e.g until the 20th day). Successively, the tumor growth was evident in all groups without significant differences among the groups (data not shown); in addition, the tumor mass obtained in 20 days in the mice from groups 3 was less solid than the tumor mass obtained in the other groups. The Figure 5 reports the values of RMA cell growth at the day 10th from the beginning of the treatment with oATP.

No significant differences were observed between the body weights measured during the different treatments. Our data show that the local treatment with 0.5 and 1 mg oATP is efficient in significantly slowing the tumor growth. In parallel, our in vitro data suggest that elevated concentrations of oATP (1 mg for about 70,000 RMA cells) are able to induce the apoptosis of these cells.

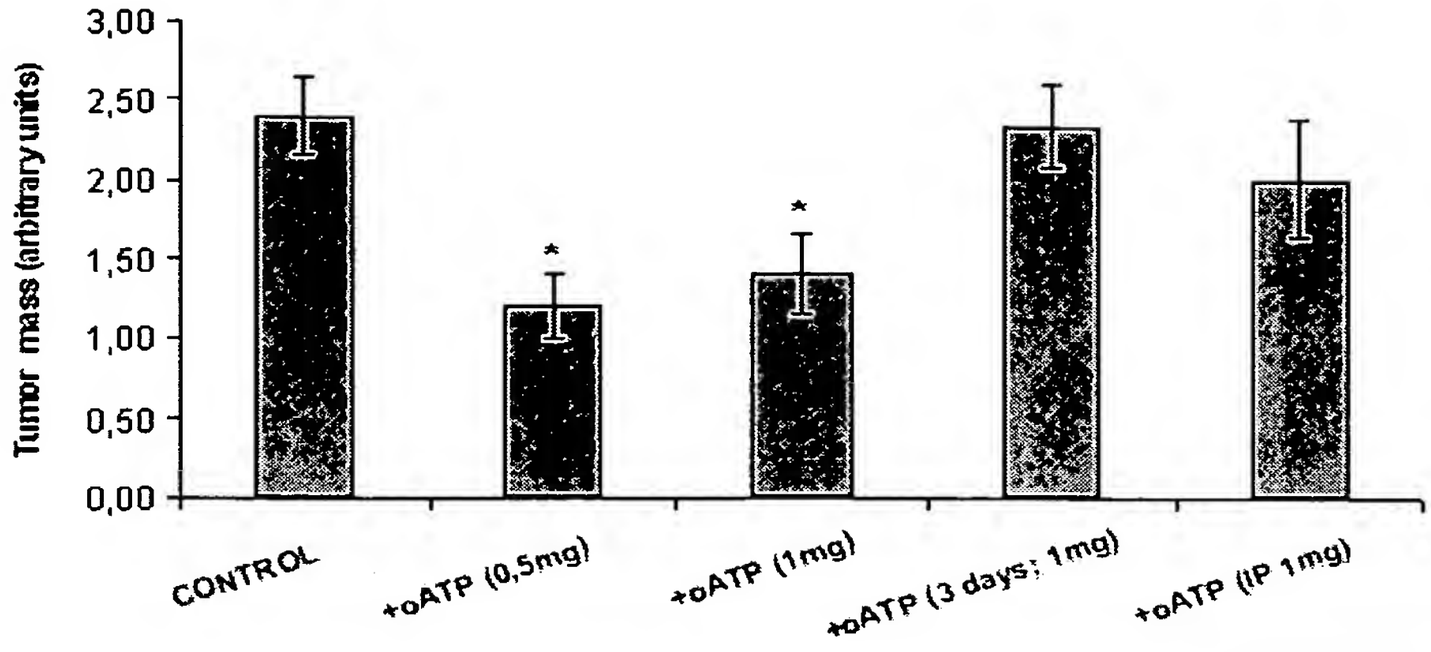


Figure 5

P < 0.05 Vs. Control

Tumor growth at the 10th day from the beginning of oATP treatment.

Thus, in view of the data show above evidencing that o-ATP is an effective antiangiogenic and antiapoptotic agent *in vitro* and *in vivo* models, applicant submits that the claimed invention is enabled.

The undersigned declare further that all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may

jeopardize the validity of the application or any patent issuing thereon.

Maria Elena Ferrero

June 10, 2008
Date